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The tail nick augments *Aeromonas sobria* serine protease (ASP) activity in plasma through retarding inhibition by α_2-macroglobulin

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ABSTRACT

ASP is a serine protease secreted by *Aeromonas sobria*, a sepsis-causing bacterium, and induces sepsis-mimicking disorders through plasma protein cleavage. The pathogen also secretes nASP that has a nick in the carboxy-terminal region. Compared with single-chain ASP (sASP), nASP had near-equivalent activity for small peptide substrates but was less proteolytic. Surprisingly, nASP cleaved proteins more in plasma and was inhibited by human α_2-macroglobulin more slowly than sASP. Retarded inhibition by α_2-macroglobulin allows nASP to keep proteolytic activity for longer in the host and exacerbate disorders at *Aeromonas sobria* infection sites. nASP may be an evolutional form to augment ASP virulence.

1. Introduction

Aeromonas species, facultative anaerobic gram-negative rods, are distributed worldwide in aquatic environments [1] and commonly cause gastroenteritis [2,3]. *Aeromonas* infections via wounds or the digestive tract often develop into systemic infections, such as peritonitis, meningitis, pneumonia and septicemia [3]. *Aeromonas* species release a number of putative virulence factors, including hemolysins, enterotoxins and proteases [4]. We purified a 65 kDa *Aeromonas sobria* serine protease [5], referred to as ASP, from culture supernatants of *A. sobria* that is most frequently isolated from patient blood [6] and is more virulent than other *Aeromonas* species [7]. ASP is a single-chain protein composed of 600 amino acid residues (sASP) and is a kexin-like serine protease belonging to the subtilisin family (subtilases) [8] (Fig. 1). The substrate specificity ASP is similar to Kex2 [9] that possesses a high degree of specificity for dibasic residues [10]. ASP releases anaphylatoxin C5a [11] from the fifth component of the complement system. C5a is a potent chemoattractant [12] that causes accumulation of neutrophils forming pus. ASP induces vascular leakage through activation of the plasma kallikrein/kinin system and lowers blood pressure [13]. ASP impairs the coagulation system by activating prothrombin [9] and degrading fibrinogen [14]. These ASP activities to induce inflammation, shock and coagulation disorder, suggest that ASP is a potent virulence factor in diseases caused by *A. sobria* infection.

This pathogen also secretes an isoform of ASP (nASP) that has a nick at the peptide bond between Gln_{519} and Leu_{520} and the cut off carboxy-terminal peptide (81 amino acid residues) is non-covalently attached to the protease body [15] (Fig. 1). As the nick is located at the junction of the protease body and the downstream ASP-specific domains is situated close to the catalytic triad Asp_{78}, His_{115} and Ser_{336} (Fig. 1), it is possible that the nick affects substrate access to the catalytic domain and ASP activity for proteins including the bait region of α_2-macroglobulin (α_2-MG), the major ASP inhibitor in human plasma [16]. α_2-MG inhibits ASP immediately after cleavage of the bait region by the protease [17], thus alteration of ASP activity may change inhibition efficiency of the inhibitor for ASP, modifying ASP activity in vivo. To investigate effects of the nick on ASP, we compared nASP with sASP in protease activity and inhibition by α_2-MG.

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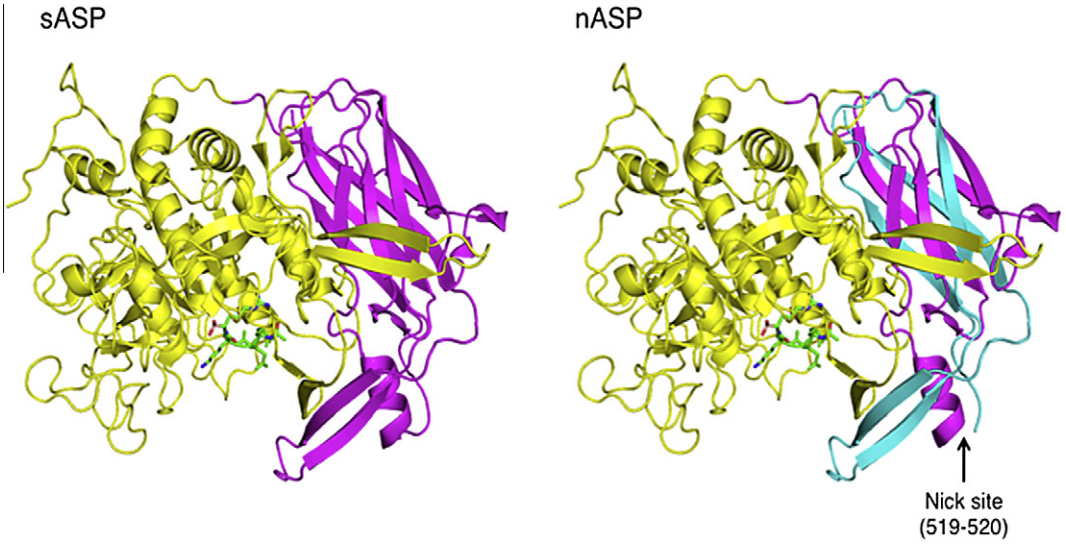

Fig. 1. Overall structure of sASP and nASP. The N-terminal domain including the catalytic site and the C-terminal P-domain are shown in yellow and purple, respectively. The catalytic site is shown with a bound substrate model (Lys-Lys-Glu) (green sticks). The C-terminal fragment of 81 amino acid residues from the nick in ASP is shown in cyan.

2. Materials and methods

2.1. Materials

4-Methylcoumaryl-7-amide (MCA) substrates were obtained from the Peptide Institute (Minoh, Japan). Human prothrombin and Thromborel® S were purchased from Enzyme Research Laboratories (South Bend, IN, USA) and Dade Behring (Eschborn, Germany), respectively. Human α-thrombin, fibrinogen and α_2-MG were obtained from Calbiochem (San Diego, CA, USA). Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan). Normal human plasma was prepared by centrifugation of a mixture of nine volumes of freshly drawn blood from healthy volunteers and one volume of 3.8% (w/v) sodium citrate.

2.2. Purification of ASP

sASP and nASP were purified from the culture supernatant of *A. sobria* strain T94 harboring pSA19-5528, as previously described [5]. Finally, sASP and nASP were purified using a hydroxyapatite column in an HPLC system and separated with a linear gradient of 1–100 mM phosphate buffer. SDS–PAGE analysis of sASP and nASP showed them to be homogeneous with molecular masses of ~64 and ~58 kDa, respectively [15]. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and amino acid sequencing analysis showed that nASP was identical to sASP, except that it was nicked between Gln_{519} and Leu_{520} [15].

2.3. Measurement of enzymatic activity

Ten microliters of MCA substrate (10 mM) was added to 600 μl of ASP solution (10 nM in 50 mM Tris–HCl, pH 7.4, containing 0.1 M NaCl) and incubated at 37 °C. In the prothrombin activation assay, 90 μl of human prothrombin at its plasma concentration (1.1 M) [18] was dissolved in 50 mM Tris–HCl, pH 7.4, containing 0.1 M NaCl and incubated with 10 μl protease at 37 °C for various periods. Then, 500 μl of 0.1 M Tris–HCl, pH 7.6, containing 150 mM NaCl was added to the mixture, followed by 10 μl of Boc-Asp(OBzl)-Pro-Arg-MCA (10 mM) [9]. The release of 7-amino-4-methyl coumarin (AMC) (fluorescence at 440 nm with excitation at 380 nm) was measured at 37 °C using a fluorescence spectrophotometer (Model 650-40, Hitachi, Tokyo, Japan), which was monitored with a recorder.

2.4. Clotting assay

Plasma coagulation time was measured with KC4Δ (Trinity Biotech, Bray, Ireland), according to published methods [9,14,19]. In the thrombin time (TT) assay, 90 μl of citrated human plasma or fibrinogen at plasma concentration [18] {3 mg/ml in 10 mM Tris–HCl, pH 7.5, containing 150 mM NaCl (TBS)} was incubated in a plastic cell with 10 μl of protease at 37 °C for 3 min. The effect of α_2-MG on fibrinogen degradation by ASP was investigated by adding 10 μl of 300 nM ASP to 90 μl of the fibrinogen mixture (3 mg/ml) with increasing concentrations of α_2-MG. Then, 100 μl human thrombin (5 U/ml) was added to initiate clotting [20,21]. In the prothrombin time (PT) assay, 45 μl of human citrated plasma and 5 μl of protease were incubated in a plastic cell at 37 °C for 3 min, followed by the addition of 50 μl Thromborel® S to initiate clotting. The controls were TBS or ASP that had been boiled for 5 min.

2.5. SDS–PAGE

ASP (70 nM) and α_2-MG (84 nM) were incubated at 37 °C, followed by the addition of 10 mM diisopropylfluorophosphate (DFP) for various time periods. Thirty microliters of each sample was mixed with 5 μl of SDS-buffer and boiled for 5 min, being analyzed by SDS–PAGE using an 8% polyacrylamide gel. A Silver Stain II kit (Wako Biochemicals, Osaka, Japan) was used for protein staining.

2.6. Fluorescence correlation spectroscopy (FCS)

Diffusion time (DT) of a fluorophore correlates its molecular weight; DT of a big molecule is longer than that of a small molecule. FCS determines DT by correlation analysis of fluorophore fluctuation. Fluorescence-labeling of ASP and FCS were performed according to the method described previously [16]. To observe ASP trapping by α_2-MG, fluorescence-labeled ASP (5 nM) was mixed with human plasma diluted fivefold with PBS and fluores-

cence at 500–530 nm was measured. Autocorrelation analysis of the fluorescence intensity fluctuation was performed using analysis software (Hamamatsu Photonics, Hamamatsu, Japan). The ratio of the larger DT value was potted chronologically and the approximation curve was obtained by polynominal fitting using MS Excel software. Velocity of complex formation of ASP with α_2-MG in plasma was estimated by the time when the ratio of the complex became 50%.

2.7. Statistics

Statistical analysis was performed using the unpaired Student's *t*-test. Values were expressed as the mean ± S.D. ($n = 4$).

3. Results

3.1. Cleavage of oligopeptide substrates by nASP

nASP protease activity was characterized by investigating the substrate specificity of nASP using various oligopeptide substrates. nASP preferentially cleaved substrates with paired basic amino acid residues at the P1 and P2 sites (Table 1), which showed that nASP has the same kexin-like protease property as sASP [9]. nASP also cleaved substrates with Arg at the P1 site, but the efficiency varied with residues at P2 and P3 sites. A preference for hydrophobic amino acid residues at the P4 site and the capability of cleaving a substrate with Phe at the P1 site [9,22,23] indicated subtilisin-like substrate specificity for nASP as sASP. nASP was unable to cleave substrates with Ala, Pro, Asp or Glu at the P1 site. The result that nASP was near-identical to sASP in substrate specificity profile and cleaving efficiency indicated a negligible effect of the nick on ASP activity for small peptide substrates.

3.2. Cleavage of proteins by nASP

Next, nASP was examined for protein cleavage. To investigate fibrinogen cleavage by nASP, an initial study tested the effect of the protease on TT. nASP prolonged fibrinogen TT in a dose- and enzymatic activity-dependent manner at concentration 10 nM or greater, while the TT prolongation activity of nASP was significantly lower than that of sASP (Fig. 2A). Interestingly, testing in plasma, the physiological source of fibrinogen, nASP prolonged the plasma TT more than sASP (Fig. 2B). Since ASP does not convert plasminogen to plasmin [18], plasmin is not involved in fibrinogen degradation by ASP in plasma. This result indicated that nASP was more fibrinogenolytic in plasma than sASP. The next experiment determined whether nASP activated prothrombin, as was found with sASP [9]. nASP generated thrombin from prothrombin but it was about 40% less effective than sASP (Fig. 3A). Prothrombin activation in plasma was investigated by measuring the PT of nASP-treated plasma. PT is a measure of the extrinsic coagulation pathway and ASP did not activate factor VII, IX or X [9], indicating that ASP shortened plasma PT solely via prothrombin activation. nASP was more potent than sASP in plasma PT shortening activity (Fig. 3B). These results suggested the greater proteolytic activity of nASP compared with sASP in plasma.

3.3. Effects of α_2-MG on nASP

Lowered proteolytic activity of nASP in comparison with sASP may be the same for cleavage of α_2-MG bait region. Lowered bait region cleavage would lead to lowered inhibition by α_2-MG and augment proteolytic activity in plasma. To compare the inhibition by α_2-MG, complex formation of ASP with α_2-MG was analyzed by SDS–PAGE. Both ASPs formed a complex with α_2-MG (Fig. 4A).

Table 1
Small substrate cleavage by single-chain ASP and nicked ASP.

		AMC release (nM min^{-1})		Ratio (nASP:sASP)
		nASP	sASP	
1	Boc-Glu-Lys-Lys-MCA	74.5	74.5	1.00
2	Pyr-Arg-Thr-Lys-Arg-MCA	61.0	63.5	0.92
3	Boc-Arg-Val-Arg-Arg-MCA	8.7	9.6	0.91
4	Boc-Leu-Lys-Arg-MCA	7.0	6.8	1.03
5	Boc-Gly-Arg-Arg-MCA	1.5	1.4	1.07
6	Boc-Ile-Glu-Gly-Arg-MCA	63.0	66.0	0.95
7	Boc-Val-Pro-Arg-MCA	7.0	7.6	0.92
8	Boc-Asp(OBzl)-Pro-Arg-MCA	4.6	4.5	1.02
9	Boc-Glu(OBzl)-Ala-Arg-MCA	3.3	3.1	1.06
10	Boc-Phe-Ser-Arg-MCA	2.1	1.8	1.17
11	Z-Phe-Arg-MCA	0	0	
12	Arg-MCA	0	0	
13	Boc-Val-Leu-Lys-MCA	5.4	4.9	1.10
14	Suc-Ala-Ala-Pro-Phe-MCA	23.1	25.5	0.91
15	Suc(OMe)-Ala-Ala-Pro-Val-MCA	0.6	0.7	0.86
16	Suc-Ala-Pro-Ala-MCA	0	0	
17	Gly-Pro-MCA	0	0	
18	Suc-Gly-Pro-Leu-Gly-Pro-MCA	0	0	
19	Ac-Asp-Glu-Val-Asp-MCA	0	0	
20	Z-Leu-Leu-Glu-MCA	0	0	

Boc, *t*-buthyloxycarbonyl; Pyr, L-pyroglutamyl; Ac, acetyl; Asp(OBzl), [–(2*S*)-2-amino-3-(benzylocarbonyl)propionyl]; Glu(OBzl), [–(2*S*)-2-amino-3-(benzylocarbonyl)butanoyl]; Z, benzyloxycarbonyl; Suc, succinyl; Suc(OMe), *N*-methoxysuccinyl.

However, nASP formed the complex more slowly and free nASP still remained after 90 s (Fig. 4A, lane h), whereas free sASP disappeared almost completely in 90 s (Fig. 4A, lane d). This demonstrated the slower inhibition of nASP by α_2-MG. The inhibitory effect of α_2-MG on ASP proteolytic activity was investigated by testing fibrinogen degradation by ASP in the presence of α_2-MG. nASP prolonged TT more than sASP at increasing α_2-MG concentrations, starting from 10 nM (Fig. 4B). α_2-MG is a thrombin inhibitor, so it may prolong TT by inhibiting the thrombin added to induce fibrin clotting. However, α_2-MG did not affect TT, even at 30 nM (data not shown). Thus, the greater effect of nASP on fibrinogen degradation under α_2-MG control suggested that nASP could keep its proteolytic activity for longer in plasma due to slower inhibition by α_2-MG.

3.4. Complex formation of nASP with α_2-MG in plasma

To investigate nASP inhibition by α_2-MG in plasma, fluorescence-labeled nASP was added to plasma and the DT was measured by FCS. The ratio of the larger DT value that corresponds to ASP-α_2-MG, in the total fluorescence value, increased time-dependently and nASP complex formation with α_2-MG was slower than sASP (Fig. 5). The 50% complex formation time estimated from the approximation curves were about 180 s in sASP and 400 s in nASP. The result suggested that nASP was inhibited by α_2-MG more than twofold slower than sASP in plasma.

4. Discussion

Comparison of nASP and sASP revealed the effects of the nick on ASP protease activity as follows: (1) essentially negligible effect on oligopeptide substrate cleavage activity (Table 1), (2) slight but significant reduction in the proteolytic activity (Figs. 2A and 3A), which matched the low prekallikrein cleavage activity of nASP compared with sASP [15], (3) increase of proteolytic activity in plasma (Figs. 2B and 3B), due to retarding inhibition by α_2-MG (Figs. 4 and 5). These results show for the first time that a bacterial protease can mitigate α_2-MG inhibition by nicking the tail region, thereby enhancing its proteolytic activity in plasma.

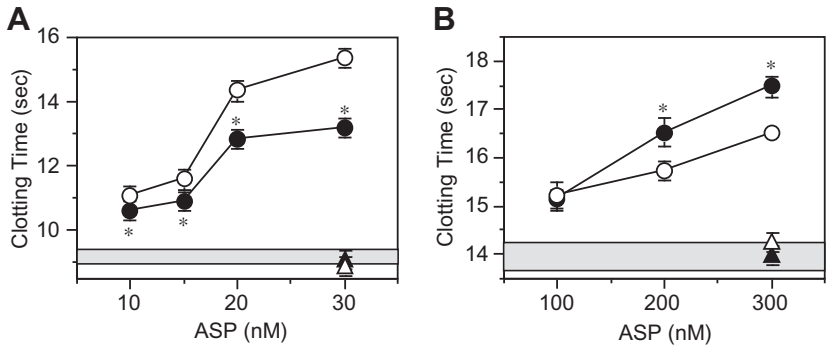

Fig. 2. ASP fibrinogen degradation measured by thrombin time (TT). Ninety microliters of human fibrinogen (3 mg/ml) (A) or citrated plasma (B) was incubated with 10 μl protease at 37 °C for 3 min, followed by the addition of 100 μl human thrombin (5 U/ml) to initiate clotting. Values are the mean ± S.D. (n = 4). Gray areas indicate the range for controls that were assayed using TBS, instead of ASP. (○), sASP; (●), nASP; (△), sASP boiled for 5 min; (▲), nASP boiled for 5 min. *p < 0.01 for nASP versus sASP.

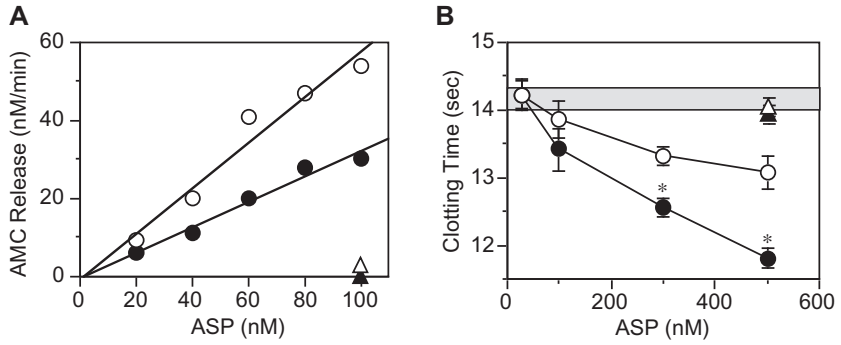

Fig. 3. Activation of prothrombin by ASP. (A) Thrombin activity assay using a MCA substrate. Ninety microliters of prothrombin (1.1 μM dissolved in 50 mM Tris–HCl, pH 7.4, containing 0.1 M NaCl and 5 mM $CaCl_2$) was incubated with 10 μl of ASP at 37 °C and then added to 500 μl of 0.1 M Tris–HCl, pH 7.6, containing 150 mM NaCl. To this mixture, 5 μl of Boc-Asp(OBzl)-Pro-Arg-MCA (10 mM) was added and the AMC released was measured. (B) Prothrombin time (PT) assay. Forty-five microliters of plasma and 5 μl of ASP were incubated at 37 °C for 3 min, followed by the addition of 50 μl of Thromborel® S to initiate clotting. Values are the mean ± S.D. (n = 4). Gray area indicates the range for controls that were assayed using TBS, instead of ASP. Symbols are the same as those used in Fig. 2. *p < 0.01 for nASP versus sASP.

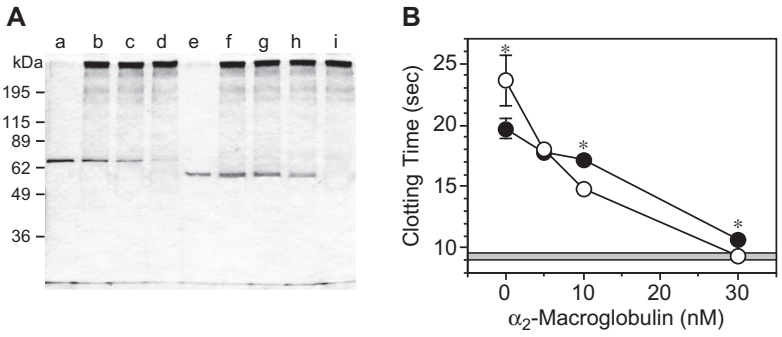

Fig. 4. A, Trapping of ASP by α_2-MG. ASP (70 nM) and α_2-MG (84 nM) were incubated at 37 °C, followed by the addition of 10 mM DFP for various time periods and analysis by SDS–PAGE using an 8% polyacrylamide gel. A Silver Stain II kit was used for protein staining. Lane a indicates sASP alone and lanes b–d indicate sASP incubated with α_2-MG for 10, 30 or 90 s, respectively. Lane e indicates nASP alone and lanes f–h indicate nASP incubated with α_2-MG for 10, 30 or 90 s, respectively. Lane i indicates α_2-MG alone. (B) Inhibition of ASP fibrinogen degradation by α_2-MG. Ten microliters of 300 nM ASP was added to 90 μl of the mixture of fibrinogen (3 mg/ml) and increasing concentrations of α_2-MG. After incubating at 37 °C for 3 min, thrombin time was measured by the addition of 100 μl of human thrombin (5 U/ml). Values are the mean ± S.D. (n = 4). Gray area indicates the range for controls that were assayed using TBS, instead of ASP. (○), sASP; (●), nASP. *p < 0.01 for nASP versus sASP.

Alteration of protease activity by nicking has been well studied in β-thrombin, which is converted from α-thrombin by cleaving off the peptide Ile_{63}-Arg_{73} in the B-chain [24]. The equivalence of β-thrombin to α-thrombin in esterase activity [25] agrees with that ASP activity for oligo-peptide substrates was essentially unchanged after being nicked (Table 1). nASP showed only a slight decrease in fibrinogen clotting activity (Fig. 2A) and a 40% loss in prothrombin activation activity (Fig. 3A), whereas the conversion of bovine α-thrombin to β-thrombin resulted in a loss of approximately 90% of fibrinogen clotting activity [26]. The cleavage site to generate β-thrombin is situated between the active site residues His_{43} and Asp_{97} in the B-chain [25], whereas the nASP nick between Gln_{519} and Leu_{520} is near the carboxy-terminus [15] (Fig. 1). Thus, the nick may less affect the site where the catalytic triad Asp_{78}, His_{115} and Ser_{336} is located. In addition, as the ASP carboxy-terminal region is embedded in the protease body [15], ASP conformation change is probably slight after being nicked. However, the flexibility of the carboxy-terminal fragment was presumably increased, which would interfere access of large molecular substrates to the catalytic domain. Indeed, the nick decreased

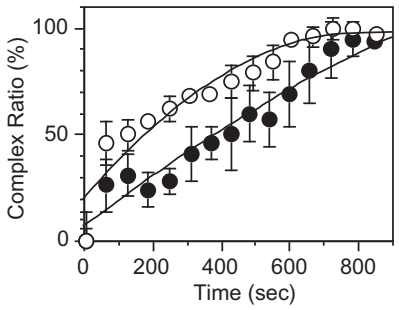

Fig. 5. Changes of α_2-MG-trapped ASP ratio in plasma. Fluorescence-labeled ASP (5 nM in PBS) was added to fivefold-diluted plasma and the ratio of fluorescence of the larger DT value was measured. Values are the mean ± S.D. (n = 4). (○), sASP; (●), nASP.

cleavage of fibrinogen and prothrombin (Figs. 2A and 3A). Contrasted with the almost unchanged k_{cat} values in the cleavage of tripeptidyl *p*-nitroanilide substrates, β-thrombin exhibited increased K_m values compared with α-thrombin [27], indicating decreased β-thrombin affinity to the substrate. Similarly, the nick of nASP may cause decrease of the substrate affinity to reduce proteolytic activity.

A protease is immediately trapped by α_2-MG when it cleaves the bait region of the inhibitor [28]. The retarded formation of α_2-MG complex with nASP (Fig. 4A) suggests slow cleavage of the bait region by nASP, which is consistent with low proteolytic activity of nASP for fibrinogen and prothrombin (Figs. 2A and 3A). α_2-MG is substantially only ASP inhibitor in human plasma [16]. Plasma containing α_2-MG leaks into interstitial tissues from the bloodstream under physiological conditions and plasma leakage increases in inflammatory lesions, including sites of *A. sobria* infections. ASP secreted from this pathogen is always under α_2-MG control. Slow inhibition by the sole regulator allows nASP to maintain its proteolytic activity for longer in plasma compared with sASP.

An *A. sobria* mutant strain that secretes non-proteolytic ASP (H115A) also releases the ASP with a nick at the same site as the nASP nick [15], indicating that nASP is not converted from sASP by autocatalysis but instead that it is produced by the activity of another *A. sobria* protease. To mitigate α_2-MG control for ASP, this pathogen might nick ASP at the tail region by its own protease, augmenting ASP proteolytic activity in the infection sites. ASP virulence is dependent on its proteolytic activity. Thus, nASP substantially elevates *A. sobria* virulence activities, such as pus formation [11], the induction of septic shock [11,13] and blood coagulation disorder [9,14], thereby exacerbating diseases caused by *A. sobria* infections. nASP may be an example of bacterial protease evolutionary change for this purpose.

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